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Immunosuppressive Cytokines (IL-10, TGF-β) Genes Expression in Human Gastric Carcinoma Tissues

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Background: Contribution of immunosuppressive cytokines to tumor progression in many types of cancers has been suggested. To characterize the in vivo expression of immunosuppressive cytokines in gastric cancer, we analyzed the messenger RNA (mRNA) expression of transforming growth factor-β (TGF-β) and interleukin-10 (IL-10) in human gastric carcinoma tissues.

Methods: Both tumor tissues and nontumor tissues from each resected specimen of 29 primary gastric carcinomas were tested for IL-10 and TGF-β mRNA expression by the reverse transcriptase-polymerase chain reaction (RT-PCR), and the mRNA expression was correlated with various pathological parameters of the tumors.

Results: Among the 29 tumors, mRNAs of TGF-β and IL-10 were detected in 79% and 62% of tumor samples, respectively. These cytokines were detected only in 31% for TGF-β and 17% for IL-10 in nontumor samples. Both mRNAs were frequently expressed in the poorly differentiated adenocarcinomas and the tumor tissues with high degree of stage or lymphnode metastasis.

Conclusions: Local expression of immunosuppressive cytokines may contribute to the progression of primary gastric carcinomas possibly through immunosuppression.

KEY WORDS: gastric carcinoma, immunosuppression, RT-PCR, TGF-β, IL-10

INTRODUCTION

Immunosuppression in patients with various advanced cancers has been well documented [1–4]. For example, decreased cellular immunoreactivity in gasric cancer patients has been reported [5]. One reason why the immune response is suppressed in cancer patients may be the production of immunosuppressive cytokines at the tumor site.

Factors released from tumor cells, which affect their growth and disrupt local homeostasis, may inhibit antitumor immunity. Interleukin-10 (IL-10) was recently found to be a potent immunosuppressive factor that can be expressed by various normal and malignant cell types of both hematopoietic and nonhematopoietic origin [6]. This cytokine has been shown to inhibit various immune functions such as cytokine production [7] and antigen-specific T-cell proliferation [8]. Transforming growth factor-β (TGF-β), another cytokine that mediates immunosuppression such as inhibiting T-cell proliferation and macrophage activation [9,10], is produced in large amount at tumor sites such as in pancreas carcinomas [11].

To obtain data that reflect in vivo expression of immunosuppressive cytokines in gastric carcinomas, we inves-

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tigated TGF-β and Il-10 mRNA expression in freshly excised tumor biopsies by the reverse transcriptase-polymerase chain reaction (RT-PCR) method using a set of cytokine-specific primers for IL-10 and TGF-β. By examining the expression of those cytokines mRNA in tumor tissues and comparing these to nontumor tissues from the same specimens, we demonstrated prevalent expression of IL-10 and TGF-β mRNA in gastric carcinoma tissues. Our results suggest that these immunosuppressive cytokines may be involved in the pathogenesis of gastric carcinomas.

MATERIALS AND METHODS

Biopsy Samples

Tumor specimens were obtained from 29 gastric cancer patients. Table I summarizes the clinical parameters of the patients whose tumors were studied. Histological analysis of the resected tumors and lymphnode was carried out in the pathological department in Kyushu University. Microscopic classification of the gastric carcinoma was performed according to the General Rules for Gastric Cancer Study of the Japanese Research Society for Gastric Cancer [12]. In the current study, there were 4 cases of well-differentiated adenocarcinoma, 7 cases of moderately differentiated adenocarcinoma, 15 cases of poorly differentiated adenocarcinoma, 2 mucinous adenocarcinomas, and 1 squamous cell carcinoma, as shown in Table I. Definite tumor tissues and nontumor tissues remote from the tumors were obtained from the same resected specimens. Parts of nontumor samples were analyzed histologically, and it was confirmed that they did not include malignant cells.

RNA Isolation, cDNA Synthesis, Polymerase Chain Reaction (PCR)

Total RNA was isolated by using the guanidium thiocyanate-phenol-chloroform single-step method [13]. The optical density at 260 nm was used to measure the concentration of total RNA, which was denatured for 15 min at 70 °C and then chilled on ice. First-strand cDNA synthesis was performed from 2 μg of RNA at 37 °C for 60 min in a final volume of 20 μl: [3 μl of denatured RNA, 4 μl of 5 × buffer (GIBCO BRL, Gaithersburg, MD), 2 μl of 100 mM dithiothreitol (BRL), 2 μl of deoxynucleotide triphosphates (dNTP) [10 mM each], 500 ng of random hexamer primers (Pharmacia, Upssala, Sweden), and 1 μl of reverse transcriptase (Superscript-RT, BRL).

PCR mixture (48 μl) was added to 2 μl of first-strand cDNA. The PCR mixture contained 5 μl of 10 × buffer [100 mM Tris-HCl/500 mM KCl/0.1% (wt/vol) gelatin, pH 8.3], 2 μl of dNTP [10 mM each] (Pharmacia), 38 μl of sterile water, 0.3 μl of Taq polymerase (Perkin-Elmer-Cetus Corp., Norwalk, CT). The reaction mixture was amplified with a thermal cycler (ASTEC, Fukuoka, Japan) for 30–35 cycles. The temperature profile used was 92°C for 1 min for denaturation, 60–65°C for 1 min for annealing, and 72°C for 1 min for primer extension. PCR products were separated on ethidium bromide-stained 1.5% agarose gel (Pharmacia). Cytokine-specific primers were synthesized on a DNA synthesizer (Biosynthesis, Lewisville, TX).

The following oligonucleotide 5′ and 3′ primer sequences were used:

TGF-β (14) 5′; AAG TGG ATC CAC GAG CCC AA
3′; GCT GCA CTT GCA GGA GCG CA
IL-10; (15) 5′; ATG CCC CAA GCT GAG AAC CAA GAC CCA
3′; TCT CAA GGG GCT GGG TCA GCT ATC CCA

PCR Products Verification and Semiquantitative Analysis by Southern Blot

To verify that PCR amplification was specific for cytokine mRNA, PCR products were tranferred to nylon filters (Hybond N, Amersham, Aylesbury, UK) and hybridized with ^{32}P-labeled probe, which were oligonucleotides complementary to sequences within the region flanked by a pair of the cytokine primers. Probes were radiolabeled with γ-^{32}P-adenine triphosphate (ICN Pharmaceuticals, Costa Mesa, CA) using T4 polynucleotide-kinase (Pharmacia) for 18 hr. Membranes were then washed for 10 min with 2 × saline-sodium citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS), followed by 0.2 × SSC and 0.1% SDS at ambient temperature, and subjected to autoradiography.

PCR products were semiquantitated using a radioanalytic imaging system (Bio Image-analyzer BAS 2000, Fujix, Tokyo, Japan). Probed membranes were scanned and the amount of radioactivity bound to PCR products present in any one lane was determined. The relative intensity of individual bands was expressed as relative count.

Statistical Analysis

Fisher's exact probability test was used for statistical analysis of cytokine gene expression. Spearman's rank correlation test was used for statistical analysis of correlation between cytokine gene expression and stage or lymphnode metastasis.

RESULTS

RT-PCR was performed to assess the expression of cytokine genes in the tumor samples and the corresponding nontumor samples from the same specimens. To rule out the possibility that any negative result was due to insufficient transcription and failure of the PCR, mRNA for the β-actin gene was examined, and the cDNA was successfully amplified in every case (data not shown). Figure 1 shows the result of a representative assay in a

TABLE I. Patient Profiles and Cytokine Expression in Gastric Carcinoma Tissues

Case	Age, sex	Stage[b]	Histology[c]	Cytokine expression[a] TGF-β	IL-10
1	65, M	III	poorly dif.	+(+)	+(−)
2	61, M	III	poorly dif.	+(−)	+(+)
3	68, M	IV	moderately dif.	+(−)	−(−)
4	71, F	II	well dif.	+(−)	−(−)
5	79, M	II	well dif.	−(−)	−(−)
6	59, F	II	poorly dif.	−(−)	−(−)
7	65, M	II	moderately dif.	−(−)	−(−)
8	79, F	III	poorly dif.	−(−)	+(−)
9	66, M	II	poorly dif.	+(−)	+(−)
10	85, M	IV	poorly dif.	+(+)	+(+)
11	46, F	IV	poorly dif.	+(−)	−(−)
12	66, M	II	well dif.	−(−)	+(−)
13	62, M	IV	well dif.	+(−)	−(−)
14	55, M	IV	poorly dif.	+(+)	+(+)
15	69, M	IV	moderately dif.	+(+)	+(−)
16	76, F	II	moderately dif.	+(+)	+(−)
17	63, M	III	poorly dif.	+(−)	+(−)
18	70, M	II	moderately	+(−)	−(−)
19	37, M	III	poorly dif.	+(−)	−(−)
20	32, F	IV	poorly dif.	+(−)	+(−)
21	67, M	II	squamous	+(−)	−(−)
22	45, M	III	moderately dif.	+(−)	+(−)
23	84, F	I	mucinous adeno.	+(+)	+(−)
24	57, M	I	moderately dif.	−(−)	−(−)
25	35, F	IV	mucinous adeno.	+(−)	+(+)
26	88, F	III	poorly dif.	+(+)	+(−)
27	45, F	III	poorly dif.	+(+)	+(−)
28	69, F	I	poorly dif.	+(−)	+(−)
29	68, M	III	poorly dif.	+(+)	+(+)

[a]Data in parentheses refer to nontumor tissues.
[b]Stage was determined according to the General Rules for Gastric Cancer Study of the Japanese Research Society for Gastric Cancer [12].
[c]Poorly dif.: poorly differentiated adenocarcinoma; moderately dif: moderately differentiated adenocarcinoma; well dif.: well differentiated adenocarcinoma; squamous: squamous carcinoma; mucinous adeno: mucinous adenocarcinoma.

sample from Patient 9. cDNA for the β-actin mRNA could be amplified in both the normal and tumor samples, whereas TGF-β and IL-10 gene transcription was detected only in cDNA derived from the tumor tissue. The characteristics of patients are summarized in Table I. cDNA for TGF-β mRNA could be amplified in 23 cases (79%) of 29 tumor samples, and in 9 cases (31%) of the corresponding 29 nontumor samples. IL-10 was found to be amplified in 18 cases (62%) of 29 tumor samples and 5 cases (17%) of the 29 nontumor samples. These results indicated that TGF-β and IL-10 mRNA were more frequently expressed in tumor samples than in nontumor samples (Table II).

To verify and semiquantify the PCR products, those were transferred to nylon membrane, hybridized with a ^{32}P-labeled oligonucleotide primer complementary to a sequence internal to the PCR amplification primers, and scanned according to β emission. Figure 2 shows a representative data from 6 patients. Prominent bands of IL-10 PCR product could be detected in 5 out of 6 tumors, but in only 1 out of 6 nontumor samples. Bands of TGF-β were positive in 5 out of 6 tumor samples, but only a faint band could be visualized in 2 out of 6 nontumor samples. Comparative analysis of the PCR products of tumor tissues and nontumor tissues after hybridization with radiolabeled specific internal probes demonstrated that the mean relative density for IL-10 was four times higher in tumor tissues than in nontumor tissues. These differences were statistically significant for IL-10 ($P = 0.001$). The TGF-β level was also four times higher in tumor tissues than in nontumor tissues. Nine nontumor samples, which were positive for TGF-β mRNA expression, were derived from advanced cancer specimens that were histologically poorly differentiated adenocarcinomas (Table I).

Differences in cytokine gene expression among tumor samples were compared among various histological types and the degree of lymphnode metastasis. It appeared that mucinous adenocarcinoma and poorly differentiated

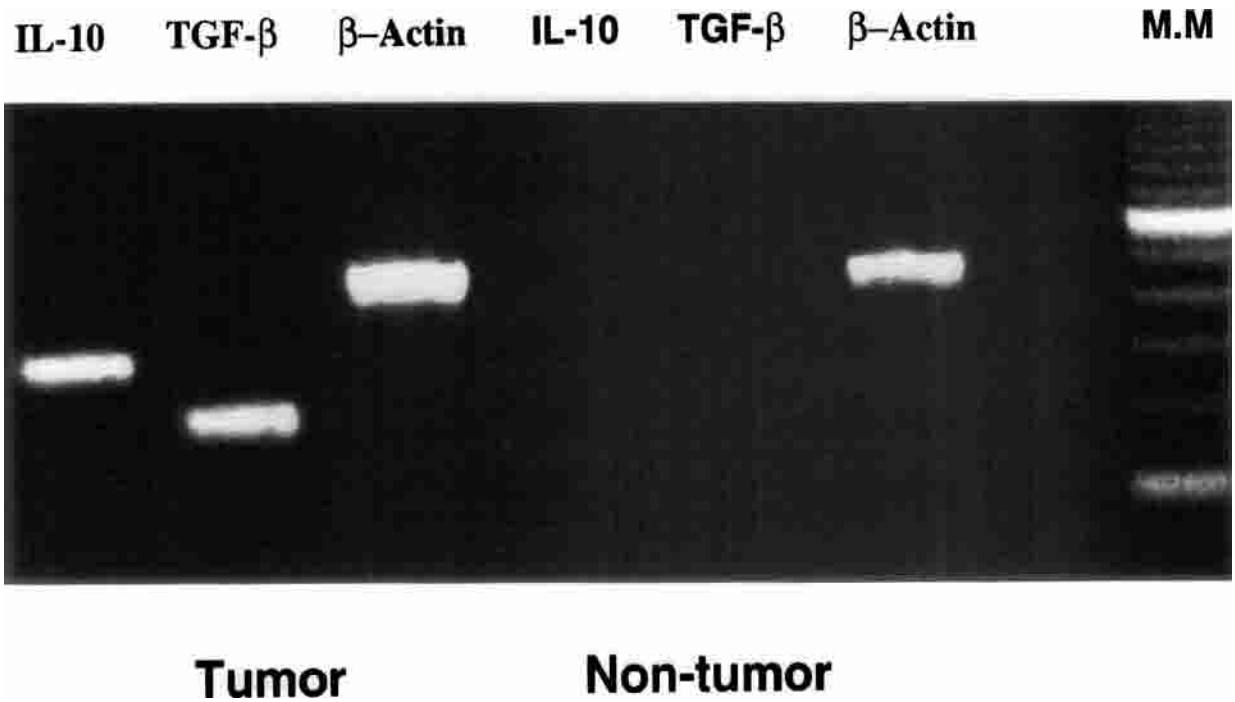

Fig. 1. Transforming growth factor-β and interleukin-10 mRNA expression determined by reverse transcriptase-polymerase chain reaction in tumor and nontumor tissues in patient 9. Total RNA, obtained from both tumor and nontumor specimens, was reverse-transcripted into cDNA. cDNA was amplified by 35 cycles polymerase chain reaction using oligonucleotide primers for β-actin, transforming growth factor-β, and interleukin-10. Polymerase chain reaction products were then run on agarose gel in the presence of ethidium bromide, and photographed under ultraviolet transillumination.

TABLE II. Transforming Growth Factor-β (TGF-β) and Interleukin-10 (IL-10) mRNA Expression in Tumor or Nontumor Tissues of Gastric Carcinomas

	β-Actin	TGF-β	IL-10
Tumor (n = 29)	29 (100%)	23 (79%)*	18 (62%)*
Nontumor (n = 29)	29 (100%)	9 (31%)	5 (17%)

* $P < 0.01$; Fisher's probability test.

carcinoma tissues expressed most frequently both cytokines (13 cases of 17 cases; 76%) (Table III). The greater the stage or degree of lymphnode metastasis were, more likely it was that both cytokines were expressed. There was a tendency for the number of samples that expressed TGF-β and IL-10 to be associated with increased node involvement and tumor stage (Tables IV, V).

DISCUSSION

Of particular importance in understanding immunosuppression in advanced cancer is the detection of immunosuppressive cytokines produced in tumor tissue. This was first accomplished by comparing the expression of IL-10 and TGF-β mRNA between tumor tissues and nontumor tissues. We used the RT-PCR technique to detect the mRNAs of those cytokines. Apparently, this assay is highly sensitive and not quantitative. Quantitative assay would give more precise results, and moreover negative results (negative expression) by PCR assay would also be meaningful, considering that the amount of transcripted cytokine gene was below the detectable level.

The present data demonstrate a distinct pattern of immuno-suppressive cytokine expression in tumor tissues. Both TGF-β and IL-10 are more frequently and abundantly expressed in tumor tissues than in adjacent nontumor tissues (Tables I and II). It seemed that poorly differentiated adenocarcinoma tissues expressed more frequently both TGF-β and IL-10 mRNAs than the others (Table III). Concomitant expression of TGF-β and IL-10 mRNA was positively correlated to stage and nodal status, of the tumor (Tables IV and V). This may be important, because the expression of immuno-suppressive cytokines mRNA may have predictive significance with respect to the pathological malignancy degree.

High levels of plasma TGF-β in patients with various tumors have been reported in breast carcinoma and hepatocellular carcinoma [16–18]. TGF-β mRNA also has been shown to be expressed more frequently in glioblastoma tumor tissues than in normal tissues [19]. Recently it has been shown that ovarian carcinomas and renal cell carcinomas express IL-10 mRNA more frequently than do noncancerous ovarian or renal tissues [20,21]. In the present study, we are the first to report the expression of IL-10 mRNA in human gastric carcinoma tissues.

We did not ascertain the cell types that were the main

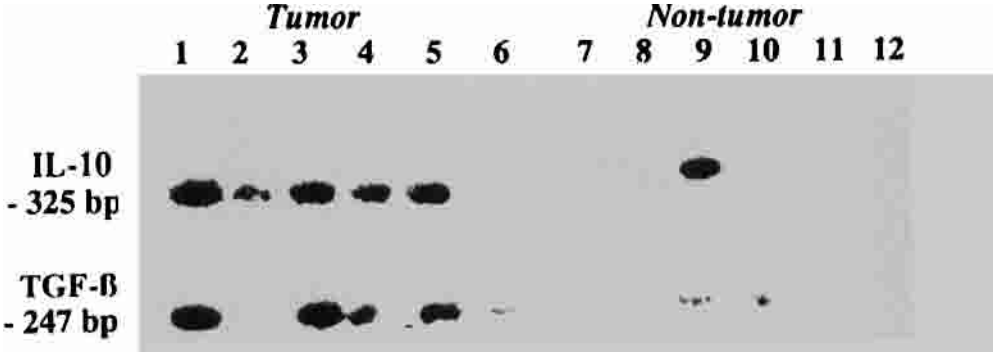

Fig. 2. Comparison of interleukin-10 and transforming growth factor-β mRNA between tumor and nontumor tissues in primary gastric carcinomas from representative six patients using reverse transcriptase-polymerase chain reaction and Southern blot analysis. RNA was extracted from freshly excised primary gastric tumor samples (lanes 1–6) and from simultaneously sampled nontumor tissues (lanes 7–12). Electrophoresed polymerase chain reaction products were probed with a radiolabeled oligonucleotide derived from a sequence internal to the primers used for the polymerase chain reaction.

TABLE III. Correlation of mRNA Expression of Transforming Growth Factor-β (TGF-β) and Interleukin-10 (IL-10) With Histological Types of Gastric Carcinoma Tissues

Histology[a]	TGF-β(−) IL-10(−)	TGF-β(+) IL-10(−)	TGF-β(−) IL-10(+)	TGF-β(+) IL-10(+)
Poorly dif. (n = 15)	1	2	1	11
Moderately diff. (n = 7)	2	2	0	3
Well dif. (n = 4)	2	1	1	0
Mucinous adeno. (n = 2)	0	0	0	2
Squamous (n = 1)	0	1	0	0

[a]Poorly dif.: poorly differentiated adenocarcinoma; moderately dif.: moderately differentiated adenocarcinoma; well dif: well differentiated adenocarcinoma; squamous: squamous cell carcinoma; mucinous adeno.: mucinous adenocarcinoma.

TABLE IV. Correlation of Transforming Growth Factor-β (TGF-β) and/or Interleukin-10 (IL-10) mRNA Expression with Stage

	Positive expression of TGF-β and/or IL-10		
Stage[a]	neither (n = 5)	either of (n = 11)	both (n = 13)
I	1	0	2
II	3	5	1
III	1	2	6
IV	0	4	4

[a]Stage was determined according to the General Rules for Gastric Cancer Study of the Japanese Research Society for Gastric Cancer [12]. $P < 0.05$; Spearman's rank correlation test was used for the statistical analysis.

TABLE V. Correlation of Transforming Growth Factor-β (TGF-β) and/or Interleukin-10 (IL-10) mRNA Expression With Lymphnode Metastasis

	Positive expression of TGF-β and/or IL-10		
N[c]	neither (n = 5)	either of (n = 11)	both (n = 13)
N0	3	7	3
N1	2	2	4
N2,3	0	2	6

[a]N (lymphnode grouping number) was determined according to the General Rules for Gastric Cancer Study in Surgery and Pathology in Japan (12). $P < 0.05$; Spearman's rank correlation test was used for the statistical analysis.

sources of these immuno-suppressive cytokines. Not only tumor cells, but also immune cells and stroma cells can produce TGF-β and IL-10. We compared lymphocyte infiltration between tumor and nontumor tissues in the same resected specimens and found no remarkable change in the number of lymphocytes infiltrating the samples (data not shown). Although it remains unclear whether tumor cells are the main source of these cytokines, tumor

tissues appear to be more abundant in immunosuppressive cytokines than do normal tissues.

Thus we suggest that TGF-β and IL-10 may contribute to the pathogenesis of gastric carcinoma, possibly through local immunosuppression.

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